

Tauroursodeoxycholic acid exerts anticholestatic effects by a cooperative cPKC α -/PKA-dependent mechanism in rat liver

R Wimmer,¹ S Hohenester,¹ T Pusch,¹ G U Denk,¹ C Rust,¹ U Beuers^{1,2}

¹ Department of Medicine II, Klinikum Grosshadern, University of Munich, Germany;

² Department of Gastroenterology & Hepatology, Academic Medical Center, University of Amsterdam, The Netherlands

Correspondence to: Professor U Beuers, Department of Gastroenterology & Hepatology, Academic Medical Center, University of Amsterdam, PO Box 22700, NL-1100 DE Amsterdam, The Netherlands; u.h.beuers@amc.uva.nl

RW and SH contributed equally to this study.

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ABSTRACT

Objective: Ursodeoxycholic acid (UDCA) exerts anticholestatic effects in part by protein kinase C (PKC)-dependent mechanisms. Its taurine conjugate, TUDCA, is a cPKC α agonist. We tested whether protein kinase A (PKA) might contribute to the anticholestatic action of TUDCA via cooperative cPKC α -/PKA-dependent mechanisms in tauro lithocholic acid (TLCA)-induced cholestasis.

Methods: In perfused rat liver, bile flow was determined gravimetrically, organic anion secretion spectrophotometrically, lactate dehydrogenase (LDH) release enzymatically, cAMP response-element binding protein (CREB) phosphorylation by immunoblotting, and cAMP by immunoassay. PKC/PKA inhibitors were tested radiochemically. In vitro phosphorylation of the conjugate export pump, Mrp2/Abcc2, was studied in rat hepatocytes and human Hep-G2 hepatoma cells.

Results: In livers treated with TLCA (10 μ mol/l)+TUDCA (25 μ mol/l), combined inhibition of cPKC by the cPKC-selective inhibitor Gö6976 (100 nmol/l) or the non-selective PKC inhibitor staurosporine (10 nmol/l) and of PKA by H89 (100 nmol/l) reduced bile flow by 36% ($p < 0.05$) and 48% ($p < 0.01$), and secretion of the Mrp2/Abcc2 substrate, 2,4-dinitrophenyl-S-glutathione, by 31% ($p < 0.05$) and 41% ($p < 0.01$), respectively; bile flow was unaffected in control livers or livers treated with TUDCA only or TLCA+taurocholic acid. Inhibition of cPKC or PKA alone did not affect the anticholestatic action of TUDCA. Hepatic cAMP levels and CREB phosphorylation as readout of PKA activity were unaffected by the bile acids tested, suggesting a permissive effect of PKA for the anticholestatic action of TUDCA. Rat and human hepatocellular Mrp2 were phosphorylated by phorbol ester pretreatment and recombinant cPKC α , nPKC ϵ , and PKA, respectively, in a staurosporine-sensitive manner.

Conclusion: UDCA conjugates exert their anticholestatic action in bile acid-induced cholestasis in part via cooperative post-translational cPKC α -/PKA-dependent mechanisms. Hepatocellular Mrp2 may be one target of bile acid-induced kinase activation.

Ursodeoxycholic acid (UDCA) has been used for the treatment of jaundice in Chinese traditional medicine since the Tang dynasty (618–907 AD) in the form of dried black bear's bile. Today, UDCA represents the only drug approved by the US Food and Drug Administration for the treatment of primary biliary cirrhosis (PBC), a model cholestatic liver disease.¹ UDCA improves biliary secretion in PBC and a number of other cholestatic disorders, such as primary sclerosing cholangitis (PSC) or intrahepatic cholestasis of pregnancy (ICP).² In early-stage PBC, UDCA delays progression to

cirrhosis^{3,4} as well as development of complications,⁵ and normalises life expectancy.^{6–8} Several mechanisms of action of UDCA have been discussed, and stimulation of impaired hepatobiliary secretion, detoxification of bile, and anti-apoptotic effects are assumed to contribute to the beneficial effect of UDCA in cholestatic disorders.⁹

UDCA conjugates, such as tauroursodeoxycholic acid (TUDCA), are potent signalling molecules both in hepatocytes and cholangiocytes.^{9,10} In hepatocytes, TUDCA has been shown to induce increases of cytosolic free calcium [Ca^{2+}]_i and Ca^{2+} influx,^{11–13} to selectively activate Ca^{2+} -dependent conventional protein kinase C α (cPKC α),^{14,15} to stimulate an integrin-dependent dual signalling pathway leading to activation of mitogen-activated protein kinases (MAPKs), Erk1/2 and p38^{MAPK},^{16–19} and to induce targeting and insertion of key apical transporters like the bile salt export pump, Bsep/Abcb11, and the conjugate export pump, Mrp2/Abcc2, into canalicular membranes of hepatocytes.^{17,20,21} In normal hepatocytes, MAPK-dependent mechanisms mediate, in part, the choleric effect of TUDCA,^{16,17} whereas in experimental cholestasis, PKC-dependent mechanisms appear to contribute to the anticholestatic action of TUDCA.^{20,22}

Tauro lithocholic acid (TLCA) is the most potent cholestatic agent among the major human bile acids²³ and has recently been shown to exert its cholestatic action at the hepatocyte level by phosphatidylinositol-3-kinase, and putatively nPKC ϵ -dependent mechanisms in isolated perfused rat livers (IPRLs) and isolated rat hepatocyte couplets.²⁴ Like TUDCA, TLCA is a potent signalling molecule which elevates hepatocellular [Ca^{2+}]_i without stimulation of Ca^{2+} influx,²⁵ selectively translocates nPKC ϵ to canalicular membranes and activates membrane-bound PKC,²⁶ and induces retrieval of key apical transporters such as the bile salt export pump, Bsep/Abcb11, from canalicular membranes of hepatocytes.²¹

Direct effects of bile acids such as TLCA or TUDCA on PKA activity in hepatocytes have not been disclosed although glucagon-induced cAMP formation was impaired by TUDCA in a staurosporine-sensitive fashion in hamster hepatocytes.²⁷ PKC agonists at moderate concentrations and PKA are known to stimulate liver cell secretion.¹⁰ A cooperative PKC-/PKA-dependent mechanism has recently been described to potentiate chloride secretion via the *Xenopus* cystic fibrosis transmembrane conductance regulator, XCFTR,²⁸ in *Xenopus* oocytes, and concomitant activation of cPKC α and PKA led to marked stimulation of insulin secretion

Table 1 Effect of the protein kinase C (PKC) inhibitors staurosporine (ST, 10 nmol/l) and Gö6976 (100 nmol/l), and the PKA inhibitor H89 (100 nmol/l) on activity of recombinant cPKC α , nPKC ϵ and PKA in vitro using myelin basic protein as kinase substrate

Inhibitor	cPKC α		nPKC ϵ		PKA	
	Concentration (nmol/min/g)	%	Concentration (nmol/min/g)	%	Concentration (pmol/min/mU)	%
H ₂ O standard	309 (74)	100	77	100	4.4 (1.7)	100
DMSO control	294 (46)	96	71 (11)	96	4.7 (1.6)	111
ST	23 (3)**	8	7 (1)**	9	1.0 (0.6)*	22
Gö6976	163 (52)	53	61 (10)	82	4.2 (1.5)	98
H89	217 (53)	71	44 (8)	58	0.2 (0.1)**	5
ST+H89	23 (4)**	8	6 (1)**	8	0.1 (0.1)**	2
Gö6976+H89	107 (20)**	35	44 (5)	60	0.4 (0.2)**	10

PKC and PKA inhibitors were tested at concentrations at least 5- to 10-fold above their elsewhere reported IC₅₀ for cPKC α and PKA, respectively, at which they did not affect bile flow and organic anion secretion in isolated perfused rat livers. For details see Materials and methods. Results are given as mean (SD) of protein kinase activity of four independent experiments (% of H₂O standard).

*p<0.05, **p<0.01 vs DMSO control (0.1%,v/v), ANOVA post hoc test (Tukey).

DMSO, dimethylsulfoxide.

in pancreatic beta cells by a convergent mechanism.²⁹ Therefore, we tested the hypothesis that TUDCA may exert anticholestatic effects in the well-established model of TLCA-induced cholestasis by a cooperative cPKC α -/PKA-dependent mechanism.

MATERIALS AND METHODS

Materials

Bile acids and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, Missouri, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was from ICN Biomedicals (Aurora, Ohio, USA). The nonselective PKC inhibitor staurosporine (ST), the cPKC inhibitor Gö6976, the PKA inhibitor H89, and the recombinant catalytic subunits of cPKC α , nPKC ϵ and PKA were from Calbiochem-Novabiochem (Nottingham, UK). The anti-MRP2/ABCC2 antibody was from Alexis (Lausen, Switzerland). A rabbit anti-pCREB antibody and a monoclonal CREB antibody were from Cell Signalling (Danvers, Massachusetts, USA), and a monoclonal mouse anti-GAPDH antibody was from Abcam (Cambridge, UK). A goat-anti-rabbit-IgG-HRP-conjugate-antibody was from Bio-Rad Lab (Munich, Germany) and a goat-anti-mouse-IgG-HRP antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). Complete protease inhibitor cocktail was from Roche Diagnostics (Mannheim, Germany). Marker molecular weight standard was from Santa Cruz Biotechnology. The Renaissance western blot chemiluminescence reagent was from NEN (Boston, Massachusetts, USA). Hyperfilm ECL was from Amersham (Little Chalfont, UK). Polyvinylidene difluoride membranes were from Millipore (Bedford, Massachusetts, USA). A cAMP enzyme immunoassay kit was from Amersham Biosciences (Freiburg, Germany). FosCholin-12 was from Anatrace (Maumee, Ohio, USA). Protein A-Sepharose was from Zymed (San Francisco, California, USA). All other chemicals were of the highest purity commercially available.

Protein kinase inhibitors

The effect of inhibitors on activity of cPKC α , nPKC ϵ , and PKA was tested in vitro at levels at least 5- to 10-fold above their IC₅₀ for cPKC α , as indicated in the literature, using myelin basic protein (MBP) as substrate to apply concentrations in the IPRs high enough to be effective, but not too high to cause cholestatic effects. The results showed that cPKC α was more effectively blocked by the nonselective PKC inhibitor staurosporine (10 nmol/l) than by the cPKC-specific inhibitor Gö6976 (10–100 nmol/l) (table 1). nPKC ϵ was inhibited by staurosporine (10 nmol/l), but not by Gö6976 (10–100 nmol/l). In addition, the selective PKA inhibitor,

H89 (100 nmol/l), and staurosporine (10 nmol/l), but not Gö6976 blocked PKA effectively (table 1).

Animals

Male Sprague–Dawley rats (208 (SD 22) g) were obtained from Charles River (Sulzfeld, Germany) and were subjected to a 12 h day–night rhythm with free access to rodent chow and water.

Isolated rat liver perfusions were performed as described in detail previously.^{20–24} Bile flow was determined gravimetrically in pretared tubes.

Perfusion protocol

Rat livers were perfused for a total of 115 min in a non-recirculating fashion with Krebs–Ringer bicarbonate solution (pH 7.4, 37°C). Twenty-five minutes after starting the perfusion, the nonselective PKC inhibitor staurosporine (final concentration in the portal vein, 10 nmol/l), the cPKC inhibitor Gö6976 (100 nmol/l), and/or the PKA inhibitor H89 (100 nmol/l) were infused continuously into the perfusion medium. Forty-five minutes after start of the perfusion, bile acids (TUDCA, TLCA, TCA, TUDCA+TLCA, TCA+TLCA), dibutyryl cAMP (dbcAMP) or the carrier DMSO only (control, 0.1%, v/v) were added continuously to the buffer by using an infusion pump to reach

Table 2 cAMP levels and protein kinase A (PKA) activity in liver tissue are not affected by the bile acids taurolithocholic acid (TLCA) and tauroursodeoxycholic acid (TUDCA)

Bile acid	Inhibitor	cAMP (pmol/g liver)	pCREB (AE)
DMSO		30.6 (3.5)	0.52 (0.07)
DMSO	ST+H89	30.1 (10.6)	0.25 (0.04)**
TUDCA		24.9 (7.7)	0.37 (0.15)
TUDCA	ST+H89	29.6 (7.4)	0.26 (0.11)
TLCA		30.8 (8.7)	0.34 (0.11)
TLCA	ST+H89	36.6 (3.3)	0.22 (0.07)
TLCA+TUDCA		31.7 (6.5)	0.36 (0.22)
TLCA+TUDCA	ST+H89	39.5 (14.0)	0.32 (0.14)

cAMP levels and phosphorylated cAMP response-element binding protein (pCREB) levels as a readout of PKA activity were determined with an enzyme-linked immunosorbent assay and by immunoblotting, respectively, in shock-frozen liver tissue after perfusion with the bile acids TLCA (10 μ mol/l) and/or TUDCA (25 μ mol/l) or their carrier, dimethyl sulfoxide (DMSO; 0.1%, v/v) in the presence or absence of the potent PKC inhibitor staurosporine (ST, 10 nmol/l) and the PKA inhibitor H89 (100 nmol/l; see fig 1 and table 1). Significant reduction of pCREB, but not cAMP in the presence of H89 in control livers depicts the specificity of action of this PKA inhibitor. For details, see Materials and methods. Mean (SD) of four to five experiments, each.

**p<0.001 vs inhibitor-free perfusion; Student t test.

final portal venous concentrations of 25 $\mu\text{mol/l}$ (TUDCA, TCA) or 10 $\mu\text{mol/l}$ (TLCA, dbcAMP). Sixty-five minutes after start of the perfusion, 1-chloro-2,4-dinitrobenzene (CDNB; 30 $\mu\text{mol/l}$), the precursor of the model Mrp2/Abcc2 substrate, 2,4-dinitrophenyl-S-glutathione (GS-DNP), was infused for 10 min. At this concentration, saturation of the biliary GS-DNP secretion was observed in the perfused rat liver.²⁰

Biliary secretion of 2,4-dinitrophenyl-S-glutathione

The model Mrp2/Abcc2 substrate, GS-DNP, was determined in bile spectrophotometrically as described previously.²⁰

Hepatovenous efflux of lactate dehydrogenase

Lactate dehydrogenase (LDH) was determined as an indicator of liver cell damage in the hepatovenous effluate by use of a standard enzymatic test.²⁰

cAMP in liver tissue

cAMP was extracted from liver tissue with a liquid phase extraction method conforming to the supplier's protocol and was determined by an enzyme immunoassay.

pCREB in liver tissue

An aliquot of shock-frozen liver tissue (-80°C) was homogenised, and proteins were separated by western blotting. pCREB and GAPDH were identified by use of specific antibodies. pCREB and GAPDH were semiquantified by densitometry and pCREB was expressed as a ratio pCREB/GAPDH.

Rat hepatocyte isolation and in vitro phosphorylation of Mrp2/Abcc2

Rat hepatocytes were isolated and cultured on collagen-coated wells as described previously.³⁰ A sample of 5×10^6 cells/well was incubated for 2 h after plating for 4 h with ^{32}P -orthophosphoric acid (74 MBq) and were then treated for 30 min with DMSO (0.1%, v/v), 100 nmol/l phorbol 12-myristate 13-acetate (PMA), 100 nmol/l PMA + 10 nmol/l staurosporine, 25 $\mu\text{mol/l}$ TUDCA, or 10 $\mu\text{mol/l}$ TLCA. Cells were then washed three times with phosphate-buffered saline (PBS).

Immunoprecipitation of rat Mrp2/Abcc2 or human MRP2/ABCC2

Rat Mrp2/Abcc2 and human MRP2/ABCC2 were immunoprecipitated from rat hepatocytes and human HepG2 hepatoma cells, respectively, as published previously.³¹ Efficacy of Mrp2/Abcc2 immunoprecipitation was tested by western blotting.

Phosphorylation of human MRP2/ABCC2 in vitro

Phosphorylation was performed using a phosphorylation buffer containing 10 $\mu\text{mol/l}$ ATP and per sample 370 kBq [γ - ^{32}P]ATP, and 400 U activated PKA or 90 ng activated cPKC α or/and 110 ng activated nPKC ϵ . MBP was used as a phosphorylation control. Samples were immunoblotted and autoradiography of the gels was performed for 18 h. The bands corresponding to ^{32}P -MRP2/ABCC2 were excised and radioactivity was counted in a scintillation counter.

Statistics

Data are expressed as mean (SD). Results were compared between different groups using ANOVA post hoc test (Tukey). Comparison of two groups only was performed using an unpaired two-tailed Student t test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Bile flow

TLCA (10 $\mu\text{mol/l}$) reduced bile flow in isolated perfused rat livers (IPRLs) to 14% of controls (figs 1A,B and 2A). TUDCA (25 $\mu\text{mol/l}$) reversed TLCA-induced inhibition of bile flow to 173% of controls. The nonselective PKC inhibitor staurosporine (10 nmol/l), the selective cPKC inhibitor Gö6976 (100 nmol/l), and the selective PKA inhibitor H89 (100 nmol/l) did not significantly affect bile flow in either control livers or in livers treated with TLCA+TUDCA (figs 1A,B and 2A). In contrast, when administered concomitantly, staurosporine+H89 as well as Gö6976+H89 induced a significant reduction of bile flow in livers treated with TLCA+TUDCA by 48% ($p < 0.01$) and 36% ($p < 0.05$), respectively, but again, did not affect bile flow in controls or livers treated with TUDCA only (figs 1A–C and 2A).

The cAMP analogue, dibutyryl cAMP (dbcAMP, 10 $\mu\text{mol/l}$), stimulated bile flow in controls by 55% ($p < 0.01$), but did not significantly enhance bile flow in livers treated with TLCA (+81%, NS) or TLCA+TUDCA (+16%, NS) indicating that stimulation of PKA only does not reverse TLCA-induced cholestasis in IPRL.

The taurine conjugate of the major human trihydroxy bile acid, cholic acid (TCA, 25 $\mu\text{mol/l}$) also reverses TLCA-induced inhibition of bile flow in rat liver.³² Unlike TUDCA, TCA does not affect hepatocellular [Ca^{2+}]_i and PKC isoform distribution at physiological concentrations.^{11–14} Indeed, reversal of TLCA-induced inhibition of bile flow by TCA was not affected by staurosporine+H89 (fig 2A) suggesting different molecular mechanisms mediating the anticholestatic action of TUDCA and TCA in TLCA-induced cholestasis.

Together, these data suggest that recovery of bile flow by TUDCA in TLCA-induced cholestasis – but not stimulation of bile flow under non-cholestatic conditions – is mediated by a cooperative cPKC-/PKA-dependent mechanism.

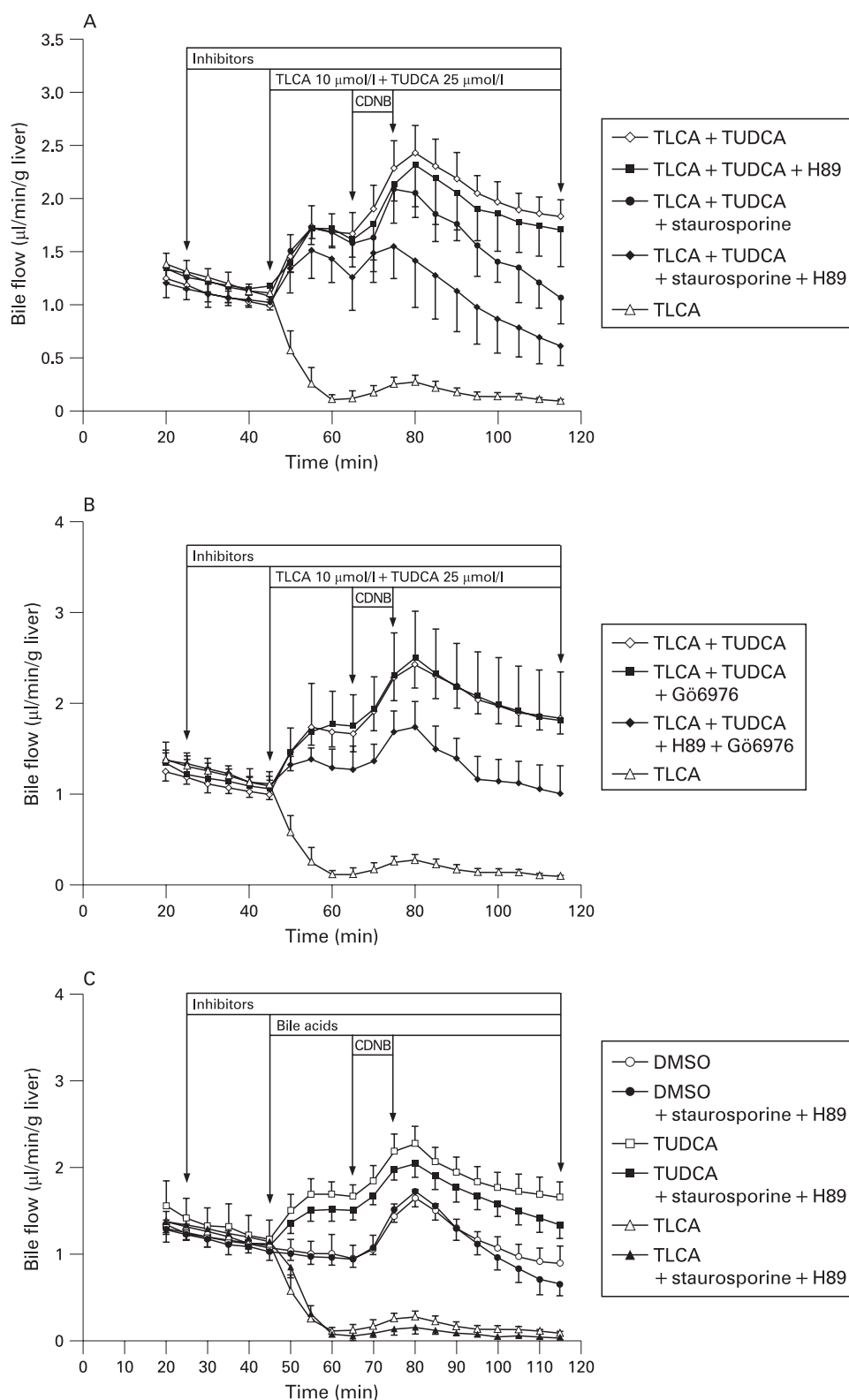
Secretion of 2,4-dinitrophenyl-S-glutathione

TLCA reduced biliary secretion of the Mrp2/Abcc2 substrate, 2,4-dinitrophenyl-S-glutathione (GS-DNP), to 7% of controls. TUDCA reversed TLCA-induced inhibition of biliary GS-DNP secretion to 113% of controls (fig 2B). The nonselective PKC inhibitor staurosporine impaired biliary GS-DNP secretion by 32% ($p < 0.05$) and the cPKC inhibitor Gö6976 tended to impair biliary GS-DNP secretion by 24% in livers treated with TLCA+TUDCA (fig 2B), but not in control livers. The PKA inhibitor H89 did not affect biliary GS-DNP secretion in livers treated with TLCA+TUDCA (fig 2B) when given alone. When administered concomitantly, staurosporine+H89 as well as Gö6976+H89 reduced biliary GS-DNP secretion by 41% ($p < 0.01$) and 31% ($p < 0.05$), respectively, in livers treated with TLCA + TUDCA. In contrast, biliary GS-DNP secretion in controls or livers treated with TUDCA only was barely affected by PKC/PKA inhibitors (fig 2B). Biliary GS-DNP secretion was not further enhanced by the cAMP analogue, dbcAMP, in controls, livers treated with TLCA or livers treated with TLCA+TUDCA (–5.7%, +1.6%, and –49%, respectively). Together, these data indicate that TUDCA-induced recovery of organic anion secretion via Mrp2/Abcc2 is mediated by a cooperative cPKC-/PKA-dependent mechanism in TLCA-induced cholestasis.

Hepatic cAMP levels and PKA activity

Hepatic cAMP levels were unaffected by any of the bile acids and inhibitors of PKC and PKA tested (table 2). In addition, CREB phosphorylation as a readout of PKA activity in liver

Figure 1 Bile flow is stimulated by tauroursodeoxycholic acid (TUDCA) via a cooperative c protein kinase C/protein kinase A (cPKC-/PKA)-dependent mechanism in tauroolithocholic acid (TLCA)-induced cholestasis, but not under control conditions. (A) Bile flow was markedly impaired by TLCA (10 $\mu\text{mol/l}$) in isolated perfused rat livers. TUDCA (25 $\mu\text{mol/l}$) reversed TLCA-induced cholestasis. The nonselective PKC antagonist staurosporine (10 nmol/l), but not the selective PKA inhibitor H89 (100 nmol/l), when given alone, tended to impair the anticholestatic effect of TUDCA whereas combined administration of staurosporine and the selective PKA inhibitor H89 significantly impaired the anticholestatic effect of TUDCA. (B) The selective cPKC inhibitor Gö6976 (100 nmol/l), when given alone, did not impair the anticholestatic effect of TUDCA in TLCA-induced cholestasis. Combined administration of Gö6976 and H89 led to a significant impairment of the anticholestatic effect of TUDCA in TLCA-induced cholestasis. (C) Combined administration of the nonselective PKC inhibitor staurosporine (10 nmol/l) and the PKA inhibitor, H89 (100 nmol/l), did neither affect bile flow of DMSO controls nor of livers treated with TUDCA or TLCA alone (see also fig 2). All results are given as means (SD) of four to five experiments, each. For statistical evaluation, see fig 2. Results for TLCA in panels B and C, and for TLCA + TUDCA in panel B are identical to those in panel A and are provided for clarity.



tissue was unaffected by the bile acids used (table 2) suggesting that PKA activity, in line with previous findings,^{10, 27} is rather permissive for the anticholestatic action of TUDCA.

Hepatovenous LDH release

The cholestatic effect of TLCA is associated with a serious cytotoxic action as demonstrated by a 32-fold increase of

hepatovenous LDH release as a readout of liver cell damage. TLCA-induced LDH release was attenuated by addition of TUDCA, but markedly increased after combined inhibition of PKC and PKA in livers treated with TLCA+TUDCA – but not after inhibition of either PKC or PKA alone – in parallel with the inhibition of the anticholestatic action of TUDCA (table 3). In contrast, LDH release was unaffected by combined inhibition of PKC and PKA in controls and livers treated with TUDCA or

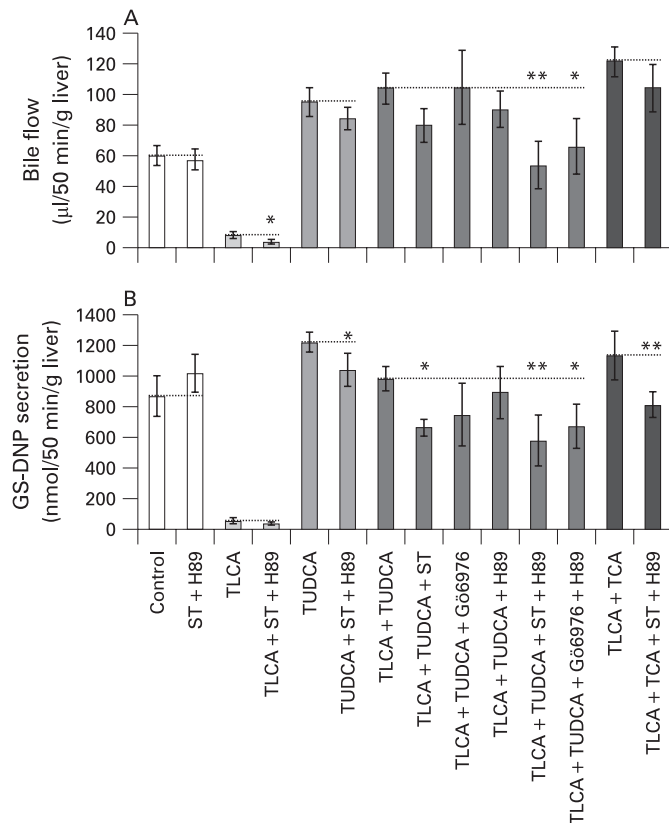


Figure 2 Bile flow and organic anion secretion are stimulated by tauroursodeoxycholic acid (TUDCA) via a cooperative c protein kinase C-/protein kinase A (cPKC-/PKA)-dependent mechanism in taurothiocholic acid (TLCA)-induced cholestasis. (A) Total bile flow during the last 50 min after addition of inhibitors, the bile acids, TLCA, TUDCA and cholic acid (TCA), and the 2,4-dinitro-S-glutathione (GS-DNP) precursor, 1-chloro-2,4-dinitrobenzene (CDNB), for the experiments shown in fig 1A,B. (B) Biliary GS-DNP secretion during the last 50 min after addition of inhibitors, the bile acids, TLCA, TUDCA and TCA, and the GS-DNP precursor CDNB. All results are given as means (SD) of four to five experiments, each. * $p < 0.05$, ** $p < 0.01$, ANOVA post hoc test.

TLCA alone (table 3). These data suggest that TUDCA alleviates TLCA-induced liver cell damage in part by a cooperative cPKC α /PKA-dependent mechanism in association with its anticholestatic action.

Phosphorylation of rat Mrp2 by the nonselective PKC agonist, phorbol 12-myristate 13-acetate

Rat Mrp2 was immunoprecipitated from freshly isolated rat hepatocytes in short-term culture after incubation of hepatocytes with 32 P-orthophosphoric acid for 4 h and with DMSO (0.1%), PMA (100 nmol/l), PMA+staurosporine (10 nmol/l), TUDCA (25 μ mol/l), and TLCA (10 μ mol/l) for 30 min. The results show that Mrp2 was phosphorylated under control conditions. The PMA-induced increase in phosphorylation was reversed by staurosporine. Bile acids at low micromolar concentrations did not affect total phosphorylation of Mrp2 (fig 3A).

Phosphorylation of human MRP2 by cPKC α , nPKC ϵ and PKA

Human MRP2³³ was immunoprecipitated from HepG2 hepatoma cells and MRP2 phosphorylation was studied in vitro using recombinant activated PKC α , PKC ϵ or PKA. The results show that the MRP2 complex after immunoprecipitation was phosphorylated by cPKC α , nPKC ϵ and PKA (fig 3B). Combined

Table 3 The cytoprotective effect of tauroursodeoxycholic acid in taurothiocholic acid-induced cholestasis is impaired by combined inhibition of c protein kinase C and protein kinase A

Bile acid	Inhibitor	LDH release	
		Concentration (mU/min/g liver)	% of controls
DMSO		2.3 (1.2)	100
DMSO	ST+H89	4.0 (5.1)	173
TUDCA		11.4 (4.4)	100
TUDCA	ST+H89	11.7 (6.8)	103
TLCA		73.1 (31.7)	100
TLCA	ST+H89	61.3 (29.3)	84
TLCA+TUDCA		21.3 (7.3)	100
TLCA+TUDCA	ST	71.8 (34.2)	336
TLCA+TUDCA	G66976	40.5 (43.3)	190
TLCA+TUDCA	H89	39.3 (7.2)	184
TLCA+TUDCA	ST+H89	95.3 (24.7)*	446
TLCA+TUDCA	G66976+H89	64.1 (53.5)	300
TLCA+TCA		2.4 (5.3)	100
TLCA+TCA	ST+H89	65.3 (52.0)	292

Hepatovenous lactate dehydrogenase (LDH) release as a readout of hepatocellular damage was determined during steady state at 85 min in perfused rat livers after addition of the bile acids TLCA (10 μ mol/l), TUDCA (25 μ mol/l), cholic acid (TCA; 25 μ mol/l), the nonselective PKC inhibitor staurosporine (ST; 10 nmol/l), the selective cPKC inhibitor G66976 (100 nmol/l), and/or the selective PKA inhibitor H89 (100 nmol/l) (see also fig 1). Results are given as mean (SD) of four to five experiments, each. Experiments with inhibitors are also given as % of respective inhibitor-free controls (100%).

* $p < 0.05$ vs respective bile acid control, ANOVA post hoc test. DMSO, dimethylsulfoxide.

incubation with cPKC α and nPKC ϵ tended to induce a more pronounced phosphorylation than incubation with cPKC α or nPKC ϵ alone in line with the assumption that different PKC isoforms induce phosphorylation of the MRP2 complex at different sites.

DISCUSSION

The present study demonstrates that the short-term anti-cholestatic effect of TUDCA in the established experimental model of TLCA-induced cholestasis is mediated mainly by a cooperative cPKC α /PKA-dependent mechanism.

We and others have previously observed that TUDCA at low micromolar concentrations stimulates Ca²⁺ entry into hepatocytes independent of inositol-1,4,5-trisphosphate,¹¹⁻¹³ selectively translocates Ca²⁺-dependent cPKC α to hepatocyte membranes,^{14 20 34} stimulates formation of *s,n*-1,2-diacylglycerol (DAG),¹⁴ activates membrane-bound PKC^{14 34} and stimulates impaired biliary secretion of organic anions and bile acids in TLCA-induced cholestasis by PKC-dependent mechanisms in isolated perfused rat liver (IPRL)²⁰ and isolated rat hepatocyte couplets,²² respectively. Similar effects of TUDCA on [Ca²⁺]_i and Ca²⁺-dependent cPKC α have been observed in cholangiocytes.³⁵ The present study not only confirms a role of PKC, but, by use of the cPKC-specific inhibitor G66976 (figs 1B and 2A,B), specifically shows that a Ca²⁺-dependent PKC isoform mediates the anticholestatic effect of TUDCA. Among the known cPKC isoforms, cPKC α , cPKC β -I, cPKC β -II and cPKC γ , only cPKC α and cPKC β -II have been detected in hepatocytes. We were not able to disclose translocation of cPKC β -II by TUDCA to hepatocyte membranes in IPRL, IRHC and Ntcp-transfected HepG2 hepatoma cells (data not shown; see also Beuers *et al*¹⁴). Thus, cPKC α is most likely the PKC isoform involved in the anticholestatic action of TUDCA in experimental cholestasis as suggested previously.^{14 20 36}

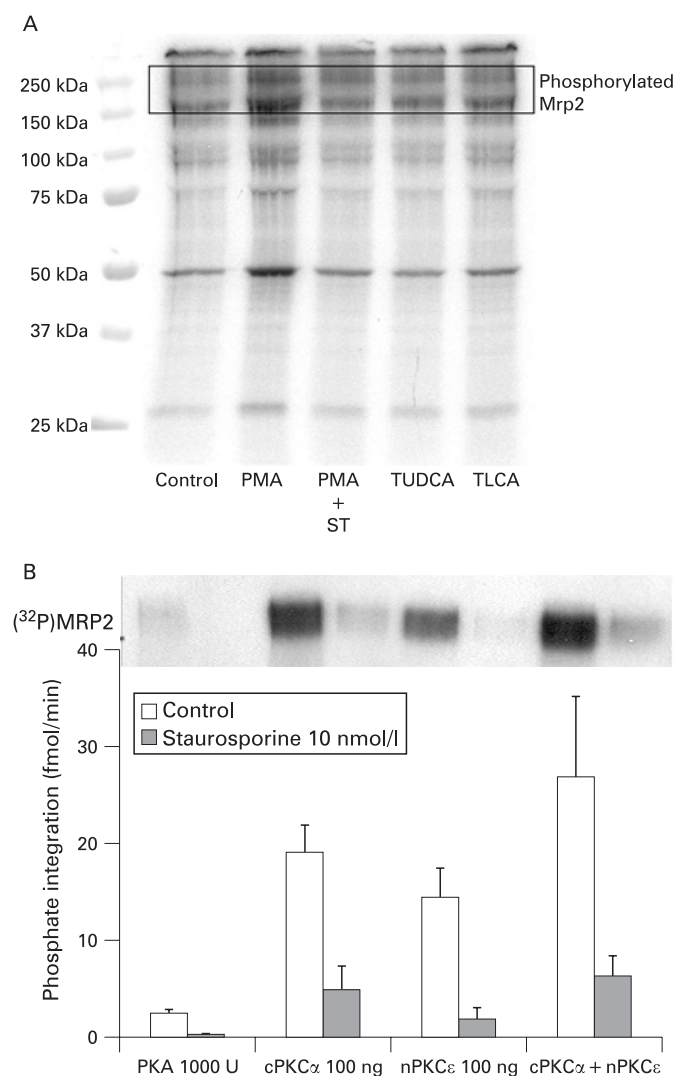


Figure 3 The conjugate export pump, MRP2/Abcc2, is phosphorylated by protein kinase C (PKC) and protein kinase A (PKA) in a staurosporine-sensitive way. (A) Rat hepatocyte total MRP2/Abcc2 phosphorylation was stimulated by phorbol 12-myristate 13-acetate (PMA), in a staurosporine-sensitive way, but not by bile acids at low micromolar concentrations. Rat hepatocytes were preloaded with ^{32}P -orthophosphoric acid and then treated for 4 h with dimethyl sulfoxide (DMSO) (0.1%, v/v), 100 nmol/l PMA, 100 nmol/l PMA + 10 nmol/l staurosporine, 25 $\mu\text{mol/l}$ tauroursodeoxycholic acid (TUDCA) or 10 $\mu\text{mol/l}$ taurothiocholic acid (TLCA). Cells were then homogenised, MRP2/Abcc2 was immunoprecipitated and immunoblotted as described in Materials and methods. A pilot experiment is shown. (B) Human MRP2/ABCC2 was phosphorylated in vitro by recombinant cPKC α , nPKC ϵ , and PKA in a staurosporine-sensitive way. MRP2/ABCC2 was immunoprecipitated from human HepG2 hepatoma cells and was exposed to recombinant cPKC α , nPKC ϵ , and PKA for 2 h in the absence or presence of the potent PKC inhibitor staurosporine (ST, 10 nmol/l). MRP2/ABCC2 phosphorylation in the presence of ^{32}P -ATP was determined radiochemically as described in Materials and methods. Results are given as means (SD) of three independent preparations.

A role of cPKC α as a mediator of the choleric effect of TUDCA in IPRL was recently questioned and the cPKC agonist thymeleatoxin was shown to induce cholestasis in IPRL.³⁷ In contrast to thymeleatoxin, TUDCA interacts with various hepatocellular (and cholangiocellular) signalling cascades.⁹ Their impact on control of diverse liver cell functions by TUDCA in health and under cholestatic conditions remains to be further

unravelling. Our previous data were in line with the assumption that bile flow and organic anion secretion under normal “non-cholestatic” conditions are modulated by TUDCA mostly independent of Ca²⁺ and Ca²⁺-dependent cPKC α (fig 1C; see also Beuers *et al*¹²) whereas Ca²⁺-dependent cPKC α appears to play a key role in the anticholestatic action of TUDCA in TLCA-induced cholestasis.²⁰

A relevant effect of TUDCA on hepatocellular PKA activity has not been observed¹⁵ although PKA is a well-known mediator of apical secretion in hepatocytes.³⁸ In line with previous findings, we were unable to observe an effect of TUDCA on cAMP levels and PKA activity as reflected by unchanged CREB phosphorylation in liver tissue. In addition, the selective PKA inhibitor H89 alone did not affect TUDCA-induced bile secretion in IPRL in the present study. It should be kept in mind, however, that global determination of CREB phosphorylation and cAMP in liver tissue may trivialise the complexity of PKA action and cAMP signals in microdomains of the apical plasma membrane.³⁹ This assumption is strengthened by the recent finding that type II inositol-1,4,5-trisphosphate receptor (InsP3) isoforms are concentrated in the pericanalicular region in a lipid raft-dependent way.^{40–41} Type II InsP3 are essential for hepatocellular Ca²⁺ wave formation and, possibly, Ca²⁺/cPKC α /PKA interaction. Thus, more sophisticated methodological approaches will be needed to further elucidate the possibly permissive role of PKA in the anticholestatic action of TUDCA in the pericanalicular zone of hepatocytes.⁹

Under non-cholestatic conditions, an integrin-dependent dual signalling pathway involving the MAPK Erk1/2 and p38^{MAPK} plays a role in mediating the choleric effect of TUDCA in IPRL.^{16–19} We were not able to show a role of MAPK for the anticholestatic action of TUDCA in TLCA-induced cholestasis when either Erk1/2 or p38^{MAPK} or Erk1/2 and p38^{MAPK} concomitantly were inhibited.³⁰ Thus, different signalling pathways mediate the choleric and the anticholestatic action of TUDCA in hepatocytes.⁹

A cooperative stimulation of secretory activity by PKC/PKA as assumed in the present model for the anticholestatic action of TUDCA has previously been described in different cell types.^{28–29} Apical carriers and their anchoring proteins^{42–45} are potential targets of a concerted action of PKA and cPKC α at the canalicular hepatocyte membrane for TUDCA-induced carrier insertion as well as of nPKC ϵ in TLCA-induced carrier retrieval.⁹ The deduced amino acid sequences of the conjugate and bile salt export pumps, MRP2/Abcc2 and Bsep/Abcb11, respectively, show numerous potential serine/threonine phosphorylation sites for PKC and PKA. We found that MRP2/Abcc2 can be phosphorylated in vitro in rat hepatocytes by PKC agonists or after immunoprecipitation from HepG2 hepatoma cells by recombinant activated cPKC α , nPKC ϵ , and PKA. Interestingly, MRP2/Abcc2 transport activity has recently been shown to be stimulated by cPKC α in a baculovirus coexpression system in vitro.⁴⁴ These findings are in line with those previously observed for Bsep/Abcb11³¹ which also can be phosphorylated by cPKC α . Identification of key serine/threonine phosphorylation sites required for apical insertion and retrieval of MRP2/Abcc2 or BSEP/ABCB11 – as demonstrated for the sodium taurocholate cotransporting polypeptide, Ntcp/Slc10a1, in the basolateral membrane⁴⁵ – will be a prerequisite to further unravel the cholestatic and anticholestatic post-translational effects of bile acids in hepatocytes.

In conclusion, the well-known anticholestatic effect of TUDCA is largely blocked by selective pharmacological intervention in the present study, indicating that TUDCA exerts its

post-translational anticholestatic effect mainly by a cooperative cPKC α -/PKA-dependent mechanism in the experimental model of TLCA-induced cholestasis.

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R Wimmer, S Hohenester, T Pusch, et al.

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